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The Role of Protein Cross-Linking in the Tumor Microenvironment

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14. ABSTRACT
In this work we have set out to answer the question of whether tissue transglutaminase is one of the enzymes that is responsible for extracellular matrix changes that correlate with tumor metastatic activity. However there is a lack of analytical tools to study and characterize what TG is doing at the molecular level. To date we have developed the necessary tools to begin to characterize TG mediated crosslinks in the extracellular environment of complex in vitro and in vivo biological systems. Future work is focus on defining how TG crosslinked matrix influences breast cancer cell lines phenotype to determine the therapeutic potential of this enzyme.

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INTRODUCTION: Cancer cells that have the ability to invade and colonize surrounding and distant (metastatic) tissues are strong indicators of poor prognosis. It is clear that the cellular microenvironment plays an important role in the progression of these deadly events. To develop synergistic therapeutic approaches that target the tumor microenvironment we need to understand, at the molecular level, the ECM's role in tumor progression. However methods are required to analyze extracellular matrix at the molecular level. This work will begin to develop the methods so that we can define the role of ECM protein and associated protein crosslinking in breast cancer progression.

The mammalian mammary gland is an extremely dynamic organ which undergoes drastic morphological changes in preparation for feeding of offspring and then again after weaning. To characterize protein changes associated with this remodeling we have used a semi-quantitative proteomics approach to identify differences in extracellular matrix (ECM) preparations from rat mammary tissue before pregnancy (nulliparous) and post weaning (involution). Involution of the mammary gland comprises a very complex and highly regulated cascade of programmed cell death and remodeling. A major component of the remodeling occurs in the ECM. Several proteases become activated in the extracellular space and there is differential ECM protein synthesis occurring in various cells within the organ. We used 1D PAGE to fractionate the isolated proteins followed by in-gel digestion of bands covering the entire gel lane. Each digestion was analyzed by RP-LC/MS/MS allowing us to identify a large number of proteins. We compared spectral counting and peak integration as two methods for semi-quantification. This GeLC-MS approach allowed us to resolve distinct molecular weight isoforms for several ECM proteins, with distinct quantifiable differences between the two ECM samples.

Tissue transglutaminase (tTG), the most significant and diverse member of the transglutaminase family, is an enzyme best known for its ability to covalently cross-link proteins. tTG plays an important role in the progression of many diseases, including cancer. tTG catalyzes the Ca2+-dependent posttranslational modification of proteins through a reaction between the gamma-carboxamide group of glutamine and the epsilon-amino group of lysine residues, to form a stable gamma-glutamyl amine cross-link. The substrates of tTG can be identified using mass spectrometry. One method to identify the reactive glutamine is to use a small molecule substitute for lysine, such as putrescine or cadaverine. If added in excess over free lysines, the reaction product after treatment with tTG will be small molecule modified substrate glutamine residues. These sites of modification can be identified using a standard "bottom-up" proteomic analysis with modification of the glutamine set as a variable for database searches. Using this approach to identify substrates of tTG from extracellular matrix preparations, we observed a shift in the precursor charge state over unmodified peptides, and as a result, lower database identification scores when using cadaverine. By using a novel small molecule, mercaptoethylamine (MEA), we were able to maintain the lower charge states observed in the unmodified peptides which increased our ability to identify the tTG modified peptides. This reagent also gives us the ability to target the modified residues for additional labeling such as biotinylation, which allows for affinity capture.

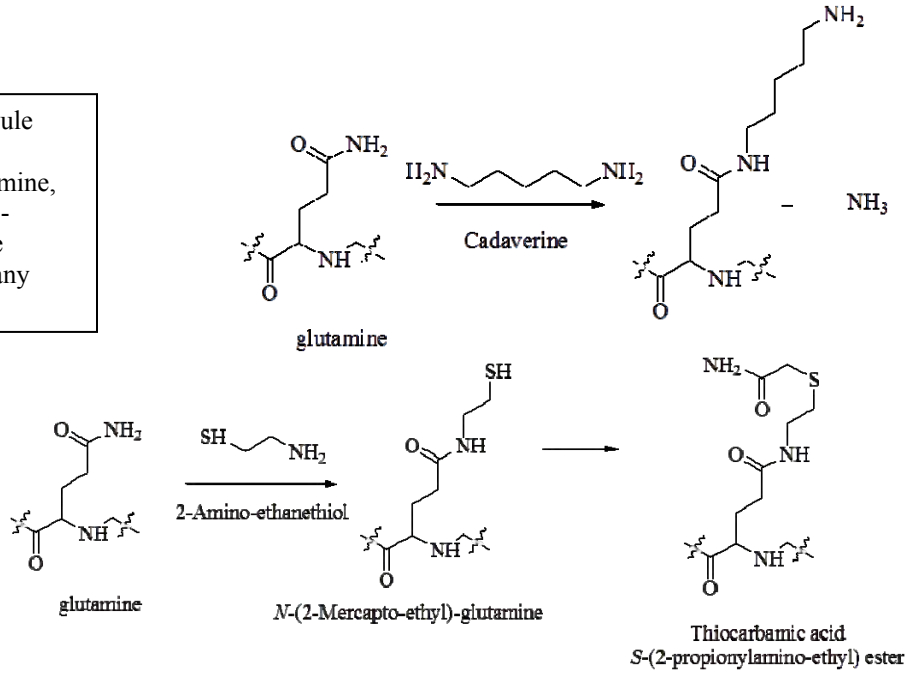
In addition, we developed a similar approach for identification of tTG reactive lysine residues using the small molecule substrate acetamide. This approach allowed us to identify a number of intracellular and extracellular protein substrates of tTG. Over 30 novel substrate sites were identified in addition to many previously reported in the international transglutaminase substrate database (Transdab Wiki). We used our findings to identify novel substrate motifs using sequence alignment.

Our ultimate goal is to measure the extent and type of tTG mediated cross-links in order to explore the role of this enzyme's involvement in cancer progression. However, the tools to accomplish this feat are not available and need to be developed. This project provides the initial tools necessary to approach our goal.

BODY:

Traditionally to identify TG reactive glutamines small molecule diamines were used as substrate (lysine substitute). Here we compare a novel method that uses 2-amino-ethanethiol (AET) to act as the substrate for TG (Figure 1.).

Figure 1. Reaction of TG with small molecule substrates for the identification of reactive glutamines. Top. Traditional use of the diamine, cadaverine. Bottom, use of new substrate 2-amino-ethanethiol that can subsequently be alkylated with iodoacetamide (shown) or any other thiol alkylating reagent.



We compared these reagents and found that the addition of a primary amine to tryptic peptides resulted in higher charge states observed in the mass spectrometry analysis. This had the detrimental effect of reducing the number of +1 and +2 charged fragment ions and as a result lower identification scores and expect values. Figure 2 shows the same peptide without modification in both of the charge states identified (+2 and +3 in this example). Modified with AET (MEA) and with cadaverine (CADV).

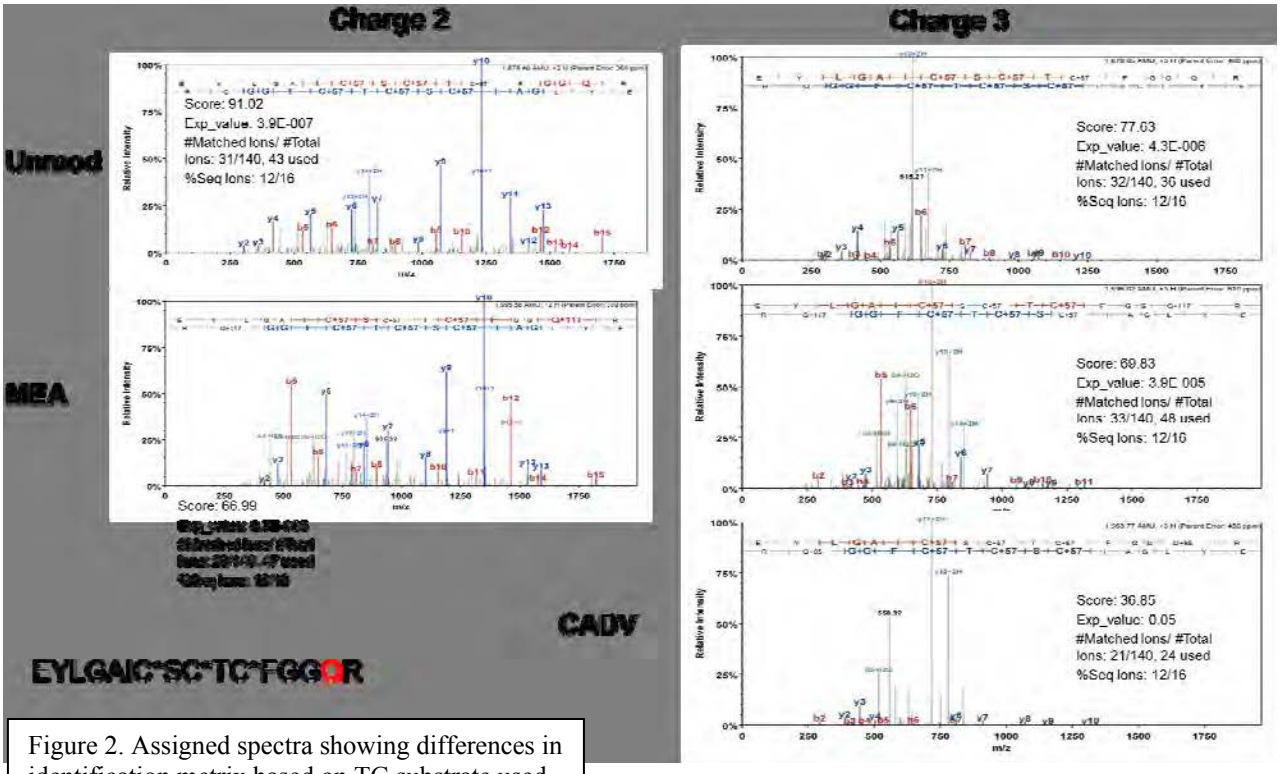
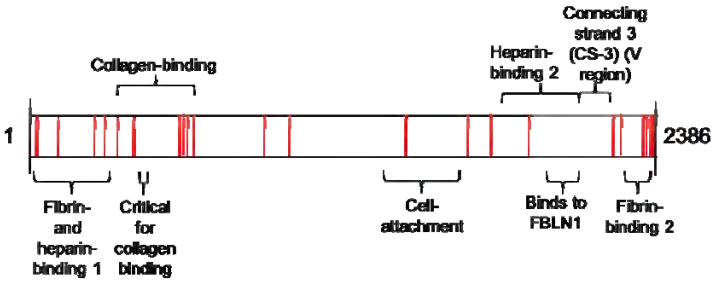


Figure 2. Assigned spectra showing differences in identification metrix based on TG substrate used for modification.

Next we performed crosslinking reactions with tTG and substrates that will be used in upcoming cell culture reactions. We analyzed the commercial basement membrane preparation Matrigel isolated from mouse EHS tumors (BD Bioscience), Collagen 1 isolated from rat tail and Fibronectin (mouse, BD Bioscience; and human, Sigma). In addition, to the two sites previously observed, we identified 33 additional sites.

Peptides
TEIDKPSQMQVTDQDNSISVK
HYQINQQWER
RPGGEPSPEGTTGQSYNQYSQR
GATYNIIVEALKDQQR
TYHVGEQWQK
WSRPQAPITGYR
GEWTCIAYSQLR
WCGTTQNYDADQK
LLCQCLGFGSGHFR
HTSVQTTSSGSGPFTDVR
EYLGAICSCTCFGGQR
QDGHLWCSTTSNYEQDQK
GNLLQCICTGNGRGEWK
EESPLLIGQQSTVSDVPR
CHEGGQSYK
IGDQWDK
IAWESPQGQVSRYR
YQCYCYGR
YHQRTNTNVNCPIECFMPLDVQADR
TETITGFQVDAVPANGQTPIQR
VDVIPVNLPGEHGQR
NRCNDQDTR

Figure 3. Using the AET reagent to map reactive Gln residues in Fibronectin, a known substrate and interaction partner of TG2



To identify in vivo crosslinks in the extracellular matrix we will first need to develop the methods required for mass spectrometry based identification. This is a very challenging task as the fragmentation pattern of the peptide will theoretically consist of fragment ions from both peptides and the crosslinked form. Standard search engines do not have the ability to consider such peptide species and as a result they will never be manually identified. For a proof-of-concept experiment we used the the small tTG substrate peptide GSQAPLR for reactions with tTG and proteins of interest. Figure 4. Show the identification of a crosslinked peptide using this approach and setting the GSQAPLR peptide as a variable modification in the database search using Protein Prospector (UCSF, NCRR MS Facility).

GSQAPLR Xlinked Peptides

m/z	z	ppm	Peptide	RT	Score	Expect	# in DB
591.3223	3	0	710.3686-AIKEGDLSTK	21.067	11.2	13	1
505.9506	3	0	710.3693-FLKNAGR	20.729	9.7	19	1
443.7438	4	0	710.3697-AIKEGDLSTK	21.147	9.5	30	1
463.2645	3	0	710.3696-AVKGFR	20.837	9.5	34	1
485.2872	3	0	710.3696-EKLVVR	22.952	8.7	53	1

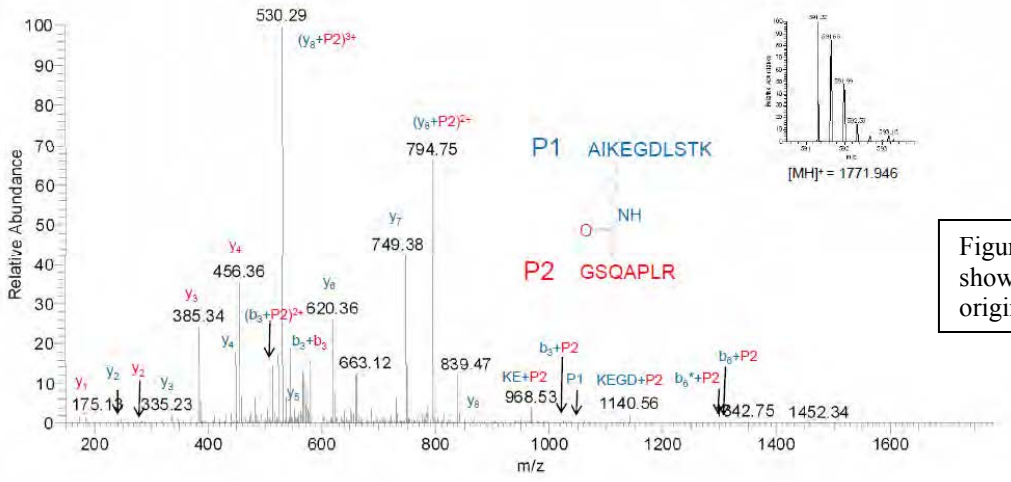


Figure 4. Assigned MS/MS spectra showing fragment ion type and originating peptide sequence.

The development of these tools has opened up the opportunity for us to begin to characterize tTG generated crosslinks in our cell culture model of crosslink mediated cell motility and invasion. Our current work involves using our tTG enzyme to modify matrix in vitro and measure cellular response (motility and invasion assays, proliferation etc.). These experiments should be completed in 3-5 months. The last goal of this work is to determine if the changes in cell phenotype are mediated through FAK signaling as part of focal adhesions. This work will follow shortly after reproducible crosslingking is achieved in our in vitro model system.

KEY RESEARCH ACCOMPLISHMENTS:

- Production of recombinant human tissue transglutaminase.
- Development of two novel activity assays – one directed at reactive lysines and one toward reactive glutamines.
- Development of a assay to determine TG substrate sites in proteins.
- Identification of reactive sites on a large number of extracellular matrix proteins including fibronectin.
- The development of a database search routine to identify crosslinked peptides from complex mixtures.

REPORTABLE OUTCOMES:

Peer Reviewed Manuscripts

Kirk C. Hansen, Lauren Kiemele, Ori Maller, Jenean O'Brien, Aarthi Shankar, Jaime Fornetti, **Pepper Schedin** "An In-Solution Ultrasonication Assisted Digestion Method for Improved Extracellular Matrix Proteome Coverage" Molecular Cellular Proteomics, 2009 Jul;8(7):1648-57.

Taraseviciute A, Vincent BT, **Schedin P**, Jones PL. Quantitative analysis of three-dimensional human mammary epithelial tissue architecture reveals a role for tenascin-C in regulating c-met function. Am J Pathol. 2010 Feb;176(2):827-38.

Rhonda Hattar, Ori Maller, Shauntae McDaniel, **Kirk C. Hansen**, Karla J. Hedman, Traci Lyons, Scott Lucia, R. Storey Wilson Jr and **Pepper Schedin** "Tamoxifen induces pleiotrophic changes in mammary stroma resulting in extracellular matrix that suppresses transformed phenotypes" Breast Cancer Research**,** 2009 Vol 11(1): R5.

Abstracts

Pepper Schedin - Information Conveyed by ECM Proteins in the Mammary Gland, Keystone Symposia on Extrinsic Control of Tumor Genesis and Progression, Vancouver, British Columbia, Mar 15, 2009.

Mammary Gland Involution Microenvironment and Tumor Progression, AACR Special Conference Advances in Breast Cancer Research: Genetics, Biology and Clinical Applications, San Diego, CA, October 15, 2009

Lauren Kiemele; Jenean O'Brien; Aarthi Shankar; Pepper Schedin; Kirk Hansen "Identification of Mammary Gland Matricryptin Candidates Using GeLC-MS/MS" Human Proteome Organization Annual International Conference, Denver, CO. 2010.

Rahul Shankar; Lauren Kiemele; Pavel Strop; Kirk Hansen "Advancing Methods for the Identification of Tissue Transglutaminase Substrates Using Mass Spectrometry" Human Proteome Organization Annual International Conference, Denver, CO. 2010.

Kirk Hansen; Lauren Kiemele; Jenean O'Brien; Pepper Schedin "Involution Induced Changes in Rat Mammary ECM Using a Label-Free GeLC-MS/MS Approach" Human Proteome Organization Annual International Conference, Denver, CO. 2010.

Presentations by Pepper Schedin at National Meetings

1. *What is tensional/ECM state of Life 1.0 and how is it different from Life 1.1? And, can the tension/ECM state be targeted to change its tumor promoting attributes?* Cancer Forum Workshop 1: Mechanical Properties of Cancer Cells & Their Microenvironment, Arizona State University, February 10, 2010
2. Meet-the-Expert Session, *Role of postpartum involution in the promotion of pregnancy-associated breast cancer,* 2010 Annual AACR Meeting, Washington, DC, April 21, 2010.
3. Phenotypic plasticity of mammary epithelial cells and implications for breast cancer development and treatment. Gordon Research Conference on Mammary Gland biology, Barga, Italy, June 13, 2010.

Patents: None to date.

Degrees obtained: None to date.

Tissue repositories: None to date.

Informatics/animal models:
Several Datasets have been generated from the proteomics work supported by this grant that are available as a resource for our and others future work.

Funding applied for and awarded based on work supported by this award:

Innovations in Cancer Sample Preparation 8/31/2010 – 8/30/2012
Principal Investigator: Kirk C. Hansen
Agency: NIH
Amount: $423,500
1 R21CA132741
Sample Preparation Methods for the Detailed Characterization of Tumor Associated Extracellular Matrix

CONCLUSION: Previous data from our lab and others has identified TG as a protein that has the potential to help define the architecture of the extracellular matrix in the mammary gland. We know that ECM architecture is changed between healthy and tumorigenic regions of the breast and that this architecture correlates with metastatic activity. We believe that TG is one of the enzymes that is responsible for this change in the matrix and as a result serves as an attractive therapeutic target. To test this hypothesis we first need tools to study and characterize what TG is doing at the molecular level. To date we have developed the necessary tools to begin to characterize TG mediated crosslinks in the extracellular environment of complex in vitro and in vivo biological systems. Future work is focus on defining how TG crosslinked matrix influences breast cancer cell lines phenotype to determine the therapeutic potential of this enzyme.

"so what section": Understanding the metastatic process is central to the development of therapies that will improve cancer patient survival. Transglutaminase (TG) modifies the extracellular matrix potentially promoting a cascade of events that increases metastatic tumor growth. Initial experiments identify TG having a role in defining the metastatic microenvironment, this identifies TG as a viable therapeutic target for the treatment and prevention of metastatic disease.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

APPENDICES: N/A